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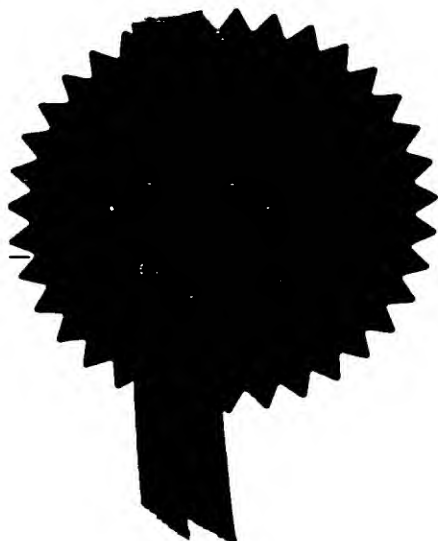
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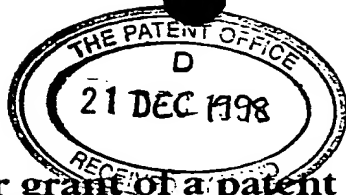
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# Request for grant of a patent

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1. Your reference

TR/RM/98102GB

2. Patent application number

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21 DEC 1998

9828201.5

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Zenco (No. 4) Limited  
15 Stanhope Gate  
London  
W1Y 5TG

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

721103000/21

4. Title of the invention

GENETIC MODIFICATION  
OF COMPOSITAE

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Batchellor, Kirk & Co.

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EC1M 5SA

Patents ADP number (if you know it)

315001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number  
(if you know it)

Date of filing  
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing  
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

Yes

- a) any applicant named in part 3 is not an inventor, or
  - b) there is an inventor who is not named as an applicant, or
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Description	10
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Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

11.

I/We request the grant of a patent on the basis of this application.

Signature

Batchellor, Kirk & Co.

Date

21.12.1998

12. Name and daytime telephone number of person to contact in the United Kingdom

Mr. D.S. Weitzel

0171 253 1563

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TITLE: GENETIC MODIFICATION OF *COMPOSITAE*

The present invention relates to the genetic modification of plants of the family *Compositae*, in particular lettuce (*Lactuca sativa*) and sunflower (*Helianthus annuus*).

Genetic modification of plants is now well established as an experimental technique, and increasingly such plants are being used in agriculture in different parts of the world. The technique offers a number of advantages. The introduction of heterologous genes allows the plant to produce heterologous proteins with functions that the plant does not normally possess. For example, introduction of the gene for production of the *Bacillus thuringiensis* (Bt) insecticidal protein renders the plant toxic to insects and protects it against insect attack. Similarly genes may be introduced which protect against the attack of fungal and viral diseases. It is also possible by genetic modification to control the expression of homologous genes which the plant already possesses. Thus by inserting extra copies of homologous genes under the control of a suitable promoter, the expression of the protein produced by such genes can be up-regulated. Correspondingly, down-regulation may be induced by means such as anti-sense technology, in which an inverted copy of the homologous gene is inserted in the plant. Expression of the inverted gene produces antisense RNA, which inhibits the expression of the natural gene. In this way, for example, ripening of fruit such as tomatoes has been delayed by inhibiting the action of the polygalacturonase gene.

The genetic modification of plants in this way offers many potential benefits to farmers, consumers and the environment. To farmers it offers the opportunity to avoid the labour and expense of chemical sprays; for consumers it can provide cheaper food of higher quality; it can protect the environment both by raising productivity (thereby

reducing pressure on agricultural land) and by reducing the amount of chemical pesticide introduced into the ecosphere.

A number of methods for transforming crop plants, both  
5 monocotyledons and dicotyledons, are now well-known. They  
include, for example, the ballistic method (the 'gene gun')  
in which heavy metal pellets, for example of gold or  
tungsten, are coated with DNA and fired into plant cells: and  
the Ti plasmid method. Another important requirement for  
10 producing useful transformed plants is the availability of an  
effective plant gene promoter. Many plant gene promoters are  
known: one very frequently used constitutive promoter is the  
CaMV (cauliflower mosaic virus) 35-S promoter. However not  
all such promoters are found to be equally effective in all  
15 plants. In some plants, in particular *Compositae*, for  
example lettuce (*Lactuca sativa*) and sunflower (*Helianthus  
annuus*), many heterologous constructs are found to have  
unstable expression levels. Both in primary transformants and  
in progeny, 'gene silencing' often causes a severe reduction  
20 in recombinant gene activity. In consequence, the plant  
reverts to a wild-type phenotype. For practical applications  
of gene technology this is unacceptable, and presents a real  
obstacle to the use of recombinant gene technology with  
plants such as lettuce and sunflower.

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According to the present invention, therefore, we  
provide a method of producing a genetically-modified  
*Compositae* plant which comprises transforming the plant with  
a heterologous DNA construct including a DNA sequence adapted  
30 to express RNA in the plant under the control of the actin2  
(ACT2) gene promoter. We further provide genetically-  
modified *Compositae* plant cells comprising a heterologous DNA  
construct including a DNA sequence adapted to express RNA in  
the plant under the control of the actin2 (ACT2) gene  
35 promoter; and *Compositae*, in particular lettuce and  
sunflower, plants comprising such cells.

The promoter of the actin2 (ACT2) gene derived from *Arabidopsis* has been shown to drive the beta-glucuronidase (GUS) gene in transgenic *Arabidopsis* plants to relatively high levels in vegetative tissues. In the inflorescence a developmental factor seemed to be involved causing a more differentiated expression pattern. This is reported by An, Meagher et al. (1996), The Plant Journal 10, 107-121: the same reference gives the DNA sequence of the actin-2 promoter (p.109).

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The invention will be further described with reference to the drawings, in which:

Figure 1 is a map of a construct pVDH380 comprising the promoter of the actin2 (ACT2) gene derived from *Arabidopsis* arranged to drive the beta-glucuronidase (GUS) gene.

Figure 2 is a map of a construct pVDH641 comprising the promoter of the actin2 (ACT2) gene derived from *Arabidopsis* arranged to drive the OXOX gene of wheat;

Figure 3 gives the DNA sequence of the actin2 (ACT2) promoter derived from *Arabidopsis*.

Actin is a fundamental cytoskeletal component essential to nearly all eukaryotic cells, in which it forms microfilament structures. There are large families of plant actin genes, with greater diversity than corresponding animal genes. The actin 2 gene promoter is a constitutive promoter to be found in most plants. We particularly prefer to use the actin 2 gene promoter obtained from *Arabidopsis thaliana*: though corresponding actin 2 promoters can readily be isolated from other sources, particularly other plants, and used for the same purpose.

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The DNA sequence which expresses RNA may be of two main kinds: either a sequence which expresses mRNA which is

translated into protein, or a sequence which produces RNA which is not translated into protein, but which interacts with the biochemistry of the plant cell in another way, for example by inhibiting gene expression.

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A preferred use of the present invention is to promote the expression of heterologous genes. However it may also be used to up-regulate or down-regulate the expression of homologous genes. As heterologous genes may be used DNA sequences coding for insecticidal proteins (for example the Bt protein) or fungicidal or antiviral proteins. ACT2 can be used to drive oxox (the oxalate oxidase gene), giving sclerotinia resistance, or other genes like early or late flowering genes (for example ATH1), herbicide resistance genes, insect tolerance genes (aphid resistance in lettuce), virus resistance genes (eg lettuce mosaic virus, LMV), nitrate reductase to lower nitrate content, and genes for increased shelf life. Many traits desirable in lettuce and sunflower can be exploited using this promoter.

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The use of DNA sequences of homologous genes to inhibit or promote gene expression is quite well understood. A complete gene sequence, under the control of a suitable promoter, that operates effectively in the plant, will generally overexpress the gene product, leading to an amplification of the effect of the protein so produced. Sometimes the gene product is reduced: this phenomenon is termed "co-suppression". Reduction of the gene product is also generally obtained by reversing the orientation of the gene sequence with respect to the promoter so that it produces "antisense" messenger RNA.

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A DNA construct for use in the invention may be an "antisense" construct generating "antisense" RNA or a "sense" construct (encoding at least part of the functional protein) generating "sense" RNA. "Antisense RNA" is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base

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(or the majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5' to 3' sense. Such antisense RNA may be  
5 produced in the cell by transformation with an appropriate DNA construct arranged to generate a transcript with at least part of its sequence complementary to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). "Sense RNA" is an  
10 RNA sequence which is substantially homologous to at least part of the corresponding mRNA sequence. Such sense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged in the normal orientation so as to generate a transcript with a sequence identical to at least  
15 part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). Suitable sense constructs may be used to inhibit gene expression (as described in International Patent Publication WO91/08299) or a sense construct encoding and expressing a homologous  
20 functional protein may be transformed into the plant to over-express the protein.

DNA constructs for use in the invention to inhibit gene expression may comprise a base sequence at least 10 bases  
25 (preferably at least 35 bases) in length for transcription into RNA. There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in  
30 length.

As a source of the DNA base sequence for transcription, a suitable cDNA or genomic DNA or synthetic polynucleotide may be used.

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The invention will be further described with reference to the following Examples.

## EXAMPLES

Lettuce was transformed with various constructs comprising the act2 gene promoter. The act2/GUS construct shown in Figure 1, kindly provided by courtesy of Dr. R. B. Meagher, Dept. of Genetics, University of Georgia, Athens, GA 30602, USA, and by us termed pVDH380 (Figure 1), was used to transform lettuce, in order to evaluate the expression pattern in primary transformants as well as the stability in consecutive generations. The construct pVDH380 was used to make a construct pVDH641 (Figure 2) containing the act2 promoter linked to the oxalate oxidase (OXOX) gene of wheat: this construct was used in transient expression studies in both lettuce and sunflower.

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## • Results

• ACT2/GUS in lettuce

The binary vector pVDH380 of Figure 1 contains the NPTII gene as a selectable marker in addition to the ACT2/GUS gene. The act2 promoter sequence includes the first 19 codons of the act2 gene as well as the first exon-intron combination. Vector pVDH380 was used directly to transform lettuce (variety "Evola"), following the Ti plasmid method given in Curtis et al. (1994), J. Exp. Bot. 45, 1441-1449.

From this transformation experiment were obtained a total of 38 independent transformants displaying a wide range of GUS-activities (as judged from a histochemical GUS staining using leaf explants of greenhouse grown material). These were compared with control CaMV 35S-GUS transformants, prepared similarly. The act2/GUS transformants of the invention showed higher and more uniform levels of GUS activity than the CaMV controls.

Subsequently, twelve independent act2/GUS transformants were used to carry out further histochemical assays. Tissues were taken from leaves, stems, roots, and flowers (sepals, petals, stamens, carpels). The tissues examined showed

consistent GUS activity levels for nine of the events. Three of the events however showed a certain degree of variability in expression (e.g. enhanced in flowers or vegetative tissues) which is probably due to the site of integration in the genome.

It was concluded that the act2/GUS construct displays a relatively strong, predominantly constitutive expression pattern in transgenic lettuce at the T0 level.

Seeds were harvested and a total number of 15 events were analyzed in the T1 generation using greenhouse grown material. The segregation data in the T1 generation, as well as levels of GUS activity in the T0 generation, are shown in Table 1 below.

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TABLE 1

Segregation analysis of transgenic lettuce expressing ACT2/GUS		
Transformant code	GUS activity in T0	Segregation of GUS activity in T1
Ev-1A-1	+	66+ / 17-
Ev-1A-3	++	20+ / 7-
Ev-2A-2	+	82+ / 24-
Ev-2A-4	-	0+ / 97-
Ev-4A-3	++	75+ / 22-
Ev-5A-2	-	0+ / 102-
Ev-5A-4	-	0+ / 97-
Ev-7A-1	-	0+ / 92-
Ev-8A-1	++	87+ / 22-
Ev-10A-1	+	75+ / 19-
Ev-10A-3	+	58+ / 20-
Ev-12A-1	+	36+ / 13-
Ev-13A-2	++	84+ / 3-
Ev-14A-1	++	78+ / 17-
Ev-14A-2	-	0+ / 98-

"+" = active; "++" = highly active; - = "not detected"

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The most important observation is that there is no significant loss of GUS activity when the gene is transferred to the T1. This contrasts sharply with the situation in which 35S-GUS is used which typically results in a total inhibition of gene activity in 90% of the events during transmission from one generation to the next.

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#### EXAMPLE 2

act2/OXOX in lettuce and sunflower

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A further experiment was done to illustrate the use of the act2 promoter to drive other genes besides GUS. As the act2/GUS construct pVDH380 (Figure 1) contains 19 codons of the actin2 gene it was decided to modify the promoter by PCR

using a primer combination which generates a unique restriction site at the act2 transcription start. This modified promoter was fused to the OXOX gene and inserted into a binary vector, as described below.

#### 5     .     Construction of ACT2-OXOX

Starting material for the ACT2-OXOX construct of Figure 2, termed pVDH641 (Figure 2), was the plasmid pVDH380 (Figure 1) which is identical with the plasmid ACT2/GUS described by An et al., cited above.

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Figure 1 shows a physical map of the construct pVDH380. In this figure, 'LB' indicates the left border, 'RB' indicates the right border, 'Pnos' indicates the nopaline synthase promoter, 'Tnos' indicates the nopaline synthase terminator, 'NPTII' indicates the neomycin phosphotransferase II gene, 'pACT2' indicates the Actin 2 promoter, 'GUS' indicates the beta-glucuronidase gene and 'KanR' indicates the bacterial kanamycin resistance gene.

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The ACT2 promoter was recloned from vector pVDH380 after amplification by PCR.

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Figure 3 shows the nucleotide sequence of the Actin 2 promoter region. The sequence corresponding to the forward primer (bold, Gothic typeface) as well as to the complementary sequence (bold underlined) of the backward primer are indicated. The start codon of the Actin 2 gene, ATG is given in bold capitals. In addition, the composition of the forward and backward primers are given. We used a primerset consisting of primer 1 (5'-GC AAGCTT ATT ATG ATC TCA AAT ACA TTG-3') and primer 2 (5'-GC GGATCC TTT ATG AGC TGC AAA CAC AC-3'). Primer 1 contains after the first two nucleotides a HindIII restriction recognition site and subsequently a nucleotide sequence identical to the nucleotide sequence located from position 1358 to position 1379 upstream from the ATG-start codon (see Figure 3). Primer 2 contains after the first two nucleotides a BamHI restriction recognition site and subsequently 20 nucleotides complementary from position 3 to position 22 upstream from the start codon. The

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DNA fragment which was obtained after amplification was digested with HindIII and BamHI and inserted in the vector pVDH478. pVDH478 is a binary vector containing between the left and right border the NPTII gene, flanked upstream by the  
5 nopaline synthase promoter and downstream by the nopaline synthase poly(A)-signal. It also contains the coding region of the oxalate oxidase gene (OxOx) with its own poly(A)-signal which is derived from wheat (for more information on the OxOx gene, including sequence data, see PCT Publication  
10 WO92/14824). The resulting vector was called pVDH641. A physical map of pVDH641 is shown in Figure 2. In this Figure, annotations in common with Figure 1 have the same meaning as in that Figure. Additionally, 'TOxOx' indicates the oxalate oxidase terminator, and 'OxOx' indicates the oxalate oxidase  
15 gene. The main restriction enzymes are indicated.

Sequence analysis confirmed that the ACT2 promoter region had been inserted without any mutation having occurred during the PCR amplification. OxOx activity can be measured in a  
20 histochemical assay using oxalate which is converted by the enzyme into a purple dye. The act2/OXOX fusion showed good levels of OxOx activity in transient assays, using both lettuce and sunflower explants, confirming the functionality of the construct.

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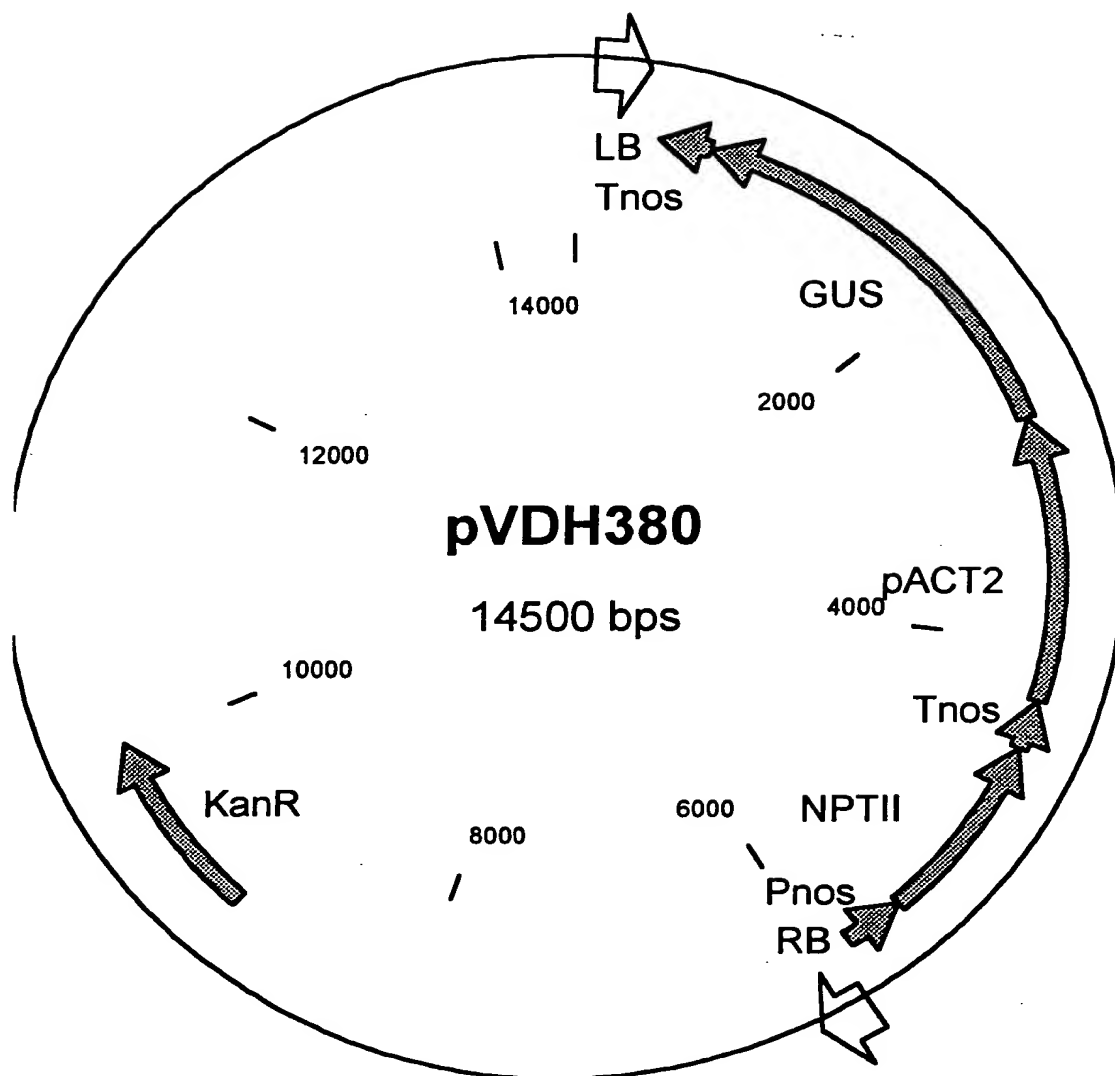


Figure 1





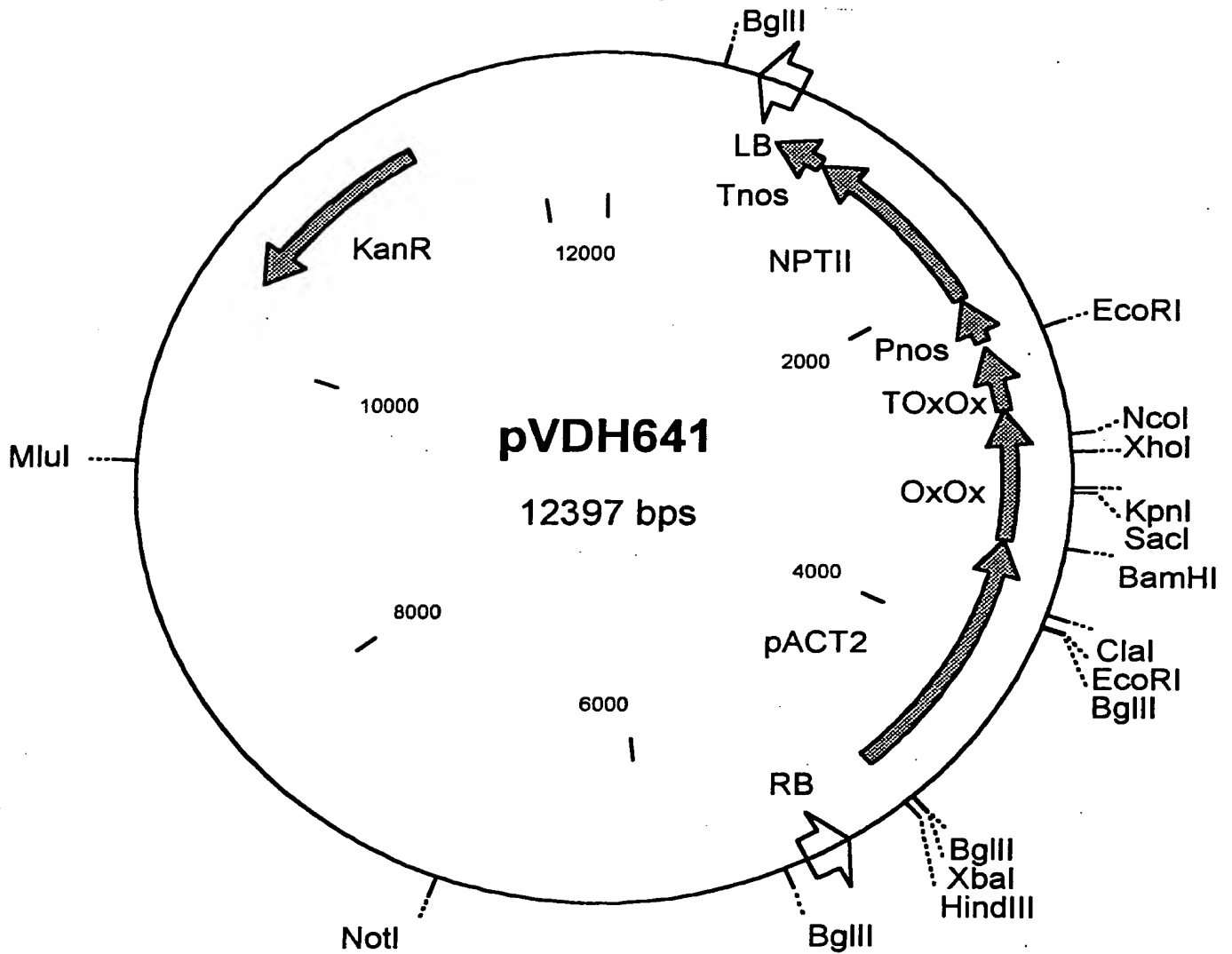


Figure 2



1 attatgact caaatacatt gatacatatc tcatctat  
 41 ctagggttatt attatgtaag aaagttttga cgaataatggn  
 81 acgacaaaat ggctacactc gatgtaattg gtatctcaac  
 121 tcaacattat acttatacca aacattagtt agcaaaattt  
 161 aaacaactat ttttatgtat gcaagagtca gcatatgtat  
 201 aattgattca gaatcgtttt gacgagttcg gatgtagtag  
 241 tagccattat ttaatgtaca tactaatcgt gaatagtgat  
 281 atgatgaaac attgtatctt attgtataaa tatccataaa  
 321 cacatcatga aagacacttt ctttcagggt ctgaattaat  
 361 tatgatacaa ttctaataga aaacgaatta aattacgttg  
 401 aattgtatga aatctaattg aacaagccaa ccacgacgag  
 441 gactaacgtt gcctggattg actcggttta agttaaccac  
 481 taaaaaaacg gagctgtcat gtaacacgcg gatcgagcag  
 521 gtcacagtca tgaagccatc aaagcaaaag aactaatcca  
 561 aggggtgaga tgattaatta gtttaaaaat tagttaacac  
 601 gagggaaaag ctgtctgaca gccaggtcac gttatcttta  
 641 cctgtggtcg aatgattcg tgtctgtcga ttttaattat  
 681 ttttttgaaa ggccgaaaat aaagttgtaa gagataaacc  
 721 cgcctatata aattcatata ttttctccc cgctttgaat  
 761 tgtctcgttg tctcctcac tttcatcagc cgttttgaat  
 801 ctccggcgac ttgacagaga agaacaagga agaagactaa  
 841 gagagaaagt aagagataat ccaggagatt cattctccgt  
 881 tttgaatctt cctcaatctc atcttcttct tccgctcttt  
 921 ctttccaagg taataggaac tttctggatc tactttattt  
 961 gctggatctc gatcttgttt tctcaatttc cttgagatct  
 1001 ggaattcgtt taatttggat ctgtgaacct ccactaaatc  
 1041 ttttggtttt actagaatcg atctaagttg accgatcagt  
 1081 tagctcgatt atagctacca gaatttggct tgaccttgat  
 1121 ggagagatcc atgttcatgt tacctgggaa atgatttgta  
 1161 tatgtgaatt gaaatctgaa ctgttgaagt tagattgaat  
 1201 ctgaacactg tcaatgttag attgaatctg aacactgttt  
 1241 aagttagatg aagtttgtgt atagattctt cgaaacctta  
 1281 ggatttgtag tgtcgtacgt tgaacagaaa gctatttctg  
 1321 attcaatcag ggtttatttg actgtattga actctttttg  
 1361 tgtgtttgca gctcataaaa aATGgctgag gctgacgata  
 1401 ttcaaccaat cgtgtgtgac aatggtactg gaatggtagg  
 1441 atcc

*HindIII*

Actin 2 primer forward. GC AAGCTT attatgact caaatacatt g

*BamHI*

Actin 2 primer backward GC GGATCC tttatgagctgcaaacacac

Figure 3

DET NO : 599 / 04317

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